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Production of Specific Copolymers of Polyhydroxyalkanoates from Industrial Waste

PHOEBY A. L. WONG,[1] HONG CHUA,[2] WAIHUNG LO,[1]
HUGH G. LAWFORD,[3] AND PETER H. YU*,[1]

[†]Open Laboratory of Chirotechnology of the Institute of Molecular Technology for Drug Discovery and Synthesis, and
[1]Department of Applied Biology & Chemical Technology,
E-mail: bcpyu@inet.polyu.edu.hk; and
[2]Department of Civil and Structure Engineering,
Hong Kong Polytechnic University, Hong Kong, China; and
[3]Department of Biochemistry, University of Toronto, Canada

Abstract

Polyhydroxyalkanoates, biodegradable plastics with the desired physical and chemical properties of conventional synthetic plastics, are extensively investigated. In this study, specific bacterial strains produced specific copolymers from food waste. Copolymers of HB and HV (poly[3-hydroxybutyrate-co-3-hydroxyvalerate]) were obtained using various ratios of butyric acid (C_4) and valeric acid (C_5) as carbon sources. The C_4 to C_5 ratio affected the melting points of the copolymers. Melting and glass transition temperatures and many other thermal properties are important parameters relative to in-service polymer applications. Higher ratios of butyrate to valerate gave higher melting points. When a mixed culture of activated sludge was employed to produce copolymers using food wastes as nutrients, the obtained copolymers showed various monomer compositions. Copolymers with a higher portion of HV were obtained using soy waste; copolymers with less HV were obtained using malt wastes. Pure strains, (i.e., *Alcaligenes latus* DSM 1122, and DSM 1124, *Staphylococcus* spp., *Klebsiella* spp.) produced specific copolymers from food waste. Only *Klebsiella* spp. produced different copolymers; the ratios of HB:HV were 93:7 and 79:21 from malt waste and soy waste, respectively. The other strains produced polymers of 100% HB. Selecting industrial food wastes as carbon sources can further reduce the cost of producing copolymers.

[†]The University Grants Committee Area of Excellence Scheme, Hong Kong

*Author to whom all correspondence and reprint requests should be addressed.

Introduction

Nondegradable, petrochemical-derived plastics accumulate in the environment at a rate of 25 million tons per year; therefore, there is much interest in creating biodegradable plastics. Currently under extensive investigation, polyhydroxyalkanoates (PHAs) are biodegradable plastics that possess the desired physical and chemical properties of conventional synthetic plastics. Poly-β-hydroxybutyrate (PHB) is the most investigated member of the PHAs. Copolymer of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) is marketed under the trade name of BIOPOL *(1)*. It is reported that PHBV is more flexible and tougher than PHB for application purposes *(2)*. Some other PHA copolymers also possess preferable properties to PHB: i.e., poly(3-hydroxybutyrate-co-3-hydroxyhexoate) *(3)*, and poly(3-hydroxybutyrate-co-3-hydroxyoctanoate) *(4)*. On the other hand, compared to the petrochemical-derived plastics, BIOPOL is much more expensive *(5)*. Much effort has been made to decrease the cost, such as using cheaper substrates *(6–9)* and improving the yield *(5)*. In our previous work, food wastes were used as a cheaper substrate to produce PHB. This also solves part of the problem of the disposal of food wastes *(6,7)*. In this article, we report on the production of specific copolymers of PHA.

Materials and Methods

Microorganism

Ralstonia eutropha (formerly *Alcaligenes eutrophus*) H16 (ATCC 17966) was purchased from American Type Culture Collection. *Alcaligenes latus* DSM 1122 and DSM 1124 were a gift from Prof. George Chen of Tsinghua University, Beijing, China. *Staphylococcus* spp. was isolated from sesame oil wastes. *Klebsiella* spp. was isolated from activated sludge. The strains were maintained on a nutrient agar slant at 4°C by monthly subculture. Mixed cultures from activated sludge were obtained from a municipal wastewater treatment plant at Shatin, Hong Kong.

Growth and Fermentation Media

Each liter of synthetic substrate medium consisted of 1 g of K_2HPO_4, 2 g $(NH_4)SO_4$, 1 g of $MgSO_4 \cdot 7H_2O$, and 0.5 g of yeast extract. Fructose or glucose (20 g) was added as a carbon source. In the production phase, the same media were used except to limit available nitrogen, and no ammonium sulfate was added. Industrial food wastes were soy wastes from Vitasoy and malt waste from Carlsberg. They were pretreated as described before *(6)*.

Food Waste Analysis: Total Organic Carbon and Total Kjeldahl Nitrogen

The procedure for total organic carbon (TOC) and total Kjeldahn nitrogen (TKN) analysis was according to APHA(4500-Norg & 5310C) *(10,11)*.

Fermentation

Fermentation was performed in a computer-controlled bioengineering fermentor with growth conditions set according to the different demands of the various bacterial strains.

Pretreatment of Food Wastes

Milled malt and semisolid soy wastes were oven-dried and digested with 1.0 *M* HCl at 105°C for 5 h. Samples were centrifuged to remove the residues, and the supernatant was neutralized with NaOH.

Extraction of PHAs

After fermentation, the fermentative broth was concentrated by centrifuging at 42,110*g* for 25 min. It was then washed twice and freeze dried. PHAs were extracted from the dried cells as described by Hahn et al. *(12)*.

Gas Chromatography

Samples for gas chromatography were prepared according to Braunegg et al. *(13)*. The analysis was performed on a Varian Model 3700 gas chromatograph, using a 1/8-in.-diameter Chromosorv-WAW column with 80/100 mesh size and 6-ft length (from Supolco, Bellefonte, PA). The recorder was a Shimadzu C-R5a Chromatopac. Nitrogen was the carrier gas at a flow rate of 10 mL/min. The analysis started at 110°C for 3 min, whereupon the temperature was increased to 220°C at a rate of 8(C/min. After reaching 220°C the temperature was maintained for 5 min before the analysis was terminated.

^{1}H-, ^{13}C-Nuclear Magnetic Resonance

^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) analyses were performed on a Bruker Model AVANCE DPX-400 spectrometer using a 5-mm ^{1}H/^{13}C dual probe and recorded as before *(6)*.

Measurement of Melting Point

Melting point was measured using an Electrothermal 9100 digital melting point apparatus. The temperature increase rate program was set at 10°C/min.

Differential Scanning Calorimetry

To study thermal behavior, differential scanning calorimetry (DSC) analysis was performed on a Perkin-Elmer 1020 Series DSC7 Thermal Analysis System under a nitrogen flow rate of 30 mL/min *(14)*.

Thermogravimetric Analysis

Thermogravimetric analysis was performed with a Perkin-Elmer TG7 thermogravimetry analyzer under a nitrogen flow rate of 20 mL/min and a scanning rate of 20(C/min *(15)*.

Table 1
Copolymer Produced by *R. eutropha* from Butyric and Valeric Acid

Carbon source C_4:C_5	Monomer molar ratio of copolymers HB:HV	Melting point (°C)
100:0	100:0	177.6
80:20	88:12	144.0
60:40	70:30	133.3
40:60	65:35	127.1
20:80	49:51	109.2
0:100	46:54	99.2

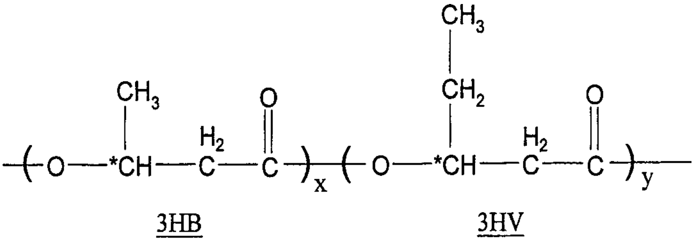

Fig. 1. Structure of poly(3-hydroxybutyrate-co-3-hydroxyvalerate).

Results and Discussion

When using various ratios of butyric acid (C_4) to valeric acid (C_5) as carbon sources, copolymers of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) with different ratios of HB:HV were obtained by *R. eutropha* (Table 1). The copolymer structure (Fig. 1) was identified from the gas chromatograph and ^1H, ^{13}C-NMR analysis. The HB:HV ratio of the copolymer was calculated from the gas chromatography analysis according to Eqs. 1 and 2, which represent, respectively, the weight calibration curves of HB and HV, determined by five different concentrations of the standard P(HB-co-HV):

$$W_{HB} = 5.869 \times (A1/A3) + 0.1908 \qquad R_1^2 = 0.9994 \tag{1}$$

$$W_{HV} = 4.608 \times (A2/A3) - 0.0118 \qquad R_2^2 = 0.9998 \tag{2}$$

in which W_{HB} is the HB weight in the sample, W_{HV} is the HV weight in the sample, A1 is the peak area of HB signal, A2 is the peak area of HV signal, and A3 is the peak area of internal standard signal. Table 1 shows that the melting point of the copolymers was affected by the C_4:C_5 ratio. As previously reported for copolymers of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate), the higher ratios of butyrate to valerate increased the melting points *(3)*. In addition, further thermal analysis of copolymers with a different HB:HV ratio was performed by DSC and thermogravimetric measurements (Table 2). From the results of DSC analysis, we

Table 2
Thermal Analysis by DSC and Thermogravimetric Measurements of Copolymers

HB:HV	DSC Analysis T_g[a] (°C)	T_c[b] (°C)	ΔH_c[c] (J/g)	T_m[d] (°C)	ΔH_m[e] (J/g)	Thermogravimetric analysis T_O[f] (°C)	T_P[g] (°C)	T_F[h] (°C)	Purity (%)
100:0	−2.29	47.43	36.06	173.1	89.54	268.85	321.95	328.3	98.6
90:10	−7.25	60.52	46.10	146.6	46.89	263.78	319.13	325.8	98.4
70:30	−8.03	65.78	50.93	133.0	42.25	258.63	317.78	323.75	98.0

[a] Glass transition temperature.
[b] Crystallizing temperature.
[c] Crystallizing enthalpy of fusion.
[d] Melting temperature.
[e] Melting enthalpy of fusion
[f] T_O temperature at the onset of weight loss.
[g] T_P temperature at the maximum rate of weight loss.
[h] T_F temperature of complete degradation

can see that the higher the ratio of butyrate to valerate, the higher the melting temperature (T_m), the melting enthalpy (ΔH_m), and the glass transition temperature (T_g). The results of enthalpy (ΔH_m), which is related to crystallinity, indicates that copolymers with higher ratio of HV show lower crystallinity. The glass transition temperature (T_g) is defined as the temperature at which a material loses its glasslike, more rigid properties and becomes rubbery and more flexible. However, the results of crystallizing temperature (T_c) show that it increased as the HV ratio increased. From the thermogravimetric analysis, we found that the higher the HV proportion, the lower the thermal decomposition temperature of the copolymer. Because melting points and some other thermal properties are important for polymer processing, it is useful to identify clearly the composition of the copolymer and its relationship to melting point. It was also observed that the copolymer texture produced from 100% butyric acid was brittle. By contrast, the copolymer texture from 100% valeric acid was more elastic and softer than that produced using other substrates.

Compared to the cost of conventional plastic, polypropylene, the cost of P(3HB-co-3HV) is much higher: less than US$1/kg vs US$16/kg (ZENECA), respectively *(5)*. The carbon source should be considered because it accounts for 78% of the raw materials, and the cost of raw materials accounts for 48% of the total operating cost. The copolymer composition varies as a function of the organism and the carbon source. In general, when specific carbon sources are made available to the cells, the desired copolymers could be produced. However, these specific carbon sources are very expensive. Fortunately, some bacteria can accumulate specific copolymers from unrelated carbon sources *(16)*. For example, P(3HB-co-3HV) could be produced by *Corynebacterium* spp, *Rhodococcus* spp., *Haloferax* spp. using fructose, glucose, sucrose, or starch as carbon sources.

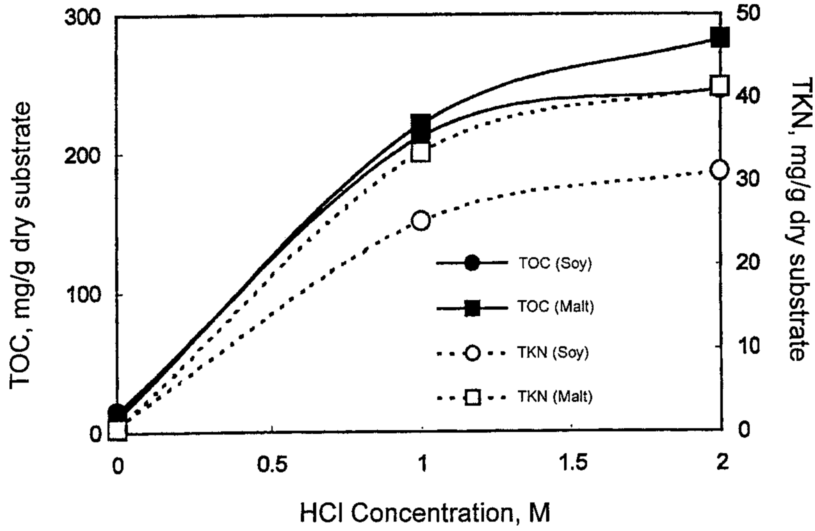

Fig. 2. TOC and TKN of malt and soy waste digested with different HCl concentrations.

Industrial food wastes still retain many useful nutrients. Figure 2 shows the TOC and TKN obtained from malt and soy waste hydrolyzed with different HCl concentrations. In addition, by using several carbohydrate tests (including Barfoed's test, Benedict's test, Bial's Orcinol-HCl test, Molisch test, Phenylhydrazine reaction, and Seliwanoff's test [17]), carbohydrate analysis of malt and soy waste could be obtained roughly. Malt waste consisted of maltose and lactose, and soy waste consisted of fructose, lactose, and maltose. Successful generation of PHB from malt and soy waste has been reported *(6,7)*.

To use malt and soy waste to produce the specific copolymers, mixed cultures of activated sludge were employed to produce copolymers. Glucose and fructose were also used as carbon sources to investigate copolymer production by activated sludge as a control to the food waste used. The obtained copolymers showed various monomer compositions (Table 3) mainly because of the mixed culture of activated sludge, a valuable biomass resource composed of a variety of types and amounts of microorganisms. The various microorganisms in activated sludge produced various copolymers when using different substrates. The HB:HV ratio of copolymer produced by the microorganisms from pure glucose and fructose feeding was 55:45 and 20:80, respectively, although the same mixed culture of activated sludge was used. The nutrients in food wastes are much more complex. However, in our study copolymers with different ratios of HB:HV were also successfully produced by a mixed culture of activated sludge from malt and soy wastes. A copolymer with more HV (HB:HV = 75:25) was obtained using soy waste as nutrients, but a copolymer with less HV (HB:HV = 90:10) was obtained from malt waste.

Table 3
Copolymers Produced by Activated Sludge from Food Wastes

Food wastes or sugars	Monomer composition of copolymer
Soy waste	75:25
Malt waste	90:10
Glucose	55:45
Fructose	20:80

Table 4
Polymers Produced from Food Waste by Pure Stains

Food waste	Strain	Monomer composition of polymer HB:HV
Malt waste	*A. latus* DSM 1122	100:0
	A. latus DSM 1124	100:0
	Staphylococcus spp.	100:0
	Klebsiella spp.	93:7
Soy Waste	*A. latus* DSM 1122	100:0
	A. latus DSM 1124	100:0
	Staphylococcus spp.	100:0
	Klebsiella spp.	79:21

Pure strains were also employed to investigate the role of strain in producing specific copolymers from food waste. *A. latus* DSM 1122 and DSM 1124, *Staphylococcus* spp., which were isolated from activated sludge, were employed. It was observed that the different HB:HV copolymers produced by *Klebsiella* spp. were 93:7 and 79:21 from malt waste and soy waste, respectively. *A. latus* DSM 1122, DSM 1124 and *Staphylococcus* spp. produced 100% HB (Table 4). Thus, specific strains should be used to produce specific copolymers from food wastes. *Klebsiella* spp. isolated from the activated sludge showed the potential to produce specific copolymers from inexpensive unrelated carbon sources. However, caution should be taken with these species because some might be pathogenic.

Using industrial food waste to produce copolymers of PHAs not only can dramatically reduce the cost of the copolymers, but also can create more novel copolymers with better physical properties.

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